Analysis of High Throughput Flow Cytometry Data using *plateCore*

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Abstract

Background

High throughput flow cytometry (FCM) studies are often run in a 96 or 384-well plate format, with different samples, controls, and antibodies-dye conjugates present on each plate. Analyzing a plate requires tracking the contents of each well, matching sample wells with control wells, gating each well/channel separately, making the appropriate plots, assessing quality, and finally aggregating the results from multiple plates to make experiment level conclusions. This process can be a significant task using traditional point-and-click software packages, even when multiple instances are deployed. We developed *plateCore* as an R/Bioconductor packaged to make processing and analysis of large, complex datasets easier.

Methods

plateCore was used to analyze the data from a BD FACS CAP screening experiment where 5 Peripheral Blood Mononucleocyte Cell (PBMC) samples were assayed for 189 different human cell surface markers. This same dataset was also manually analyzed by a cytometry expert using the FlowJo data analysis software package (TreeStar, Ashland OR).

Results

Positive markers identified using *plateCore* are in good agreement with those found using manual analysis.

Conclusions

plateCore provides a reproducible, objective platform for analyzing high throughput FCM experiments. The R/Bioconductor implementation allows bioinformaticians and statisticians access to the data, which should further the development of automated analysis methods.

Introduction

While there are a number of different software packages available for analysis of FCM data, these programs are often ill-suited to the development of new methods needed for analyzing high-throughput FCM studies. Flow Cytometry High Content Screening (FC-HCS) experiments generate large volumes of data, and a systematic approach to preprocessing, gating (i.e. filtering), and summarizing results is needed for robust analyses. Automation of these steps would allow analysis pipelines to be robust, objective, and match the high-throughput capacity of modern cytometers. Unfortunately, current approaches to FC-HCS analysis are semi-automated at best, and they often require significant subjective and error-prone manual intervention to identify cells of interest (Maecker et al., 2005). It is therefore desirable to develop programmatic approaches to process FCM data.

FCM packages available through the Bioconductor (Gentleman et al., 2004) project provide an open platform that can be used by cytometrists, bioinformaticians, and statisticians to collaboratively develop new methods for automated FC-HCS analysis. The basic data processing tools for importing, transforming, gating, and organizing raw FCM data are in the *flowCore* package (Hahne et al., 2009), and the visualization functions are in *flowViz* (Sarkar et al., 2008). The Bioconductor model for FCM data analysis facilitates the development of new analysis methods, since the overhead associated with accessing and visualizing FCM data is handled by *flowCore* and *flowViz*. The availability of *flowCore* and *flowViz* has enabled the creation of new tools for quality assessment of large FCM experiments, such as *flowQ* (Gentleman et al.), and model-based clustering and automated gating, such as *flowClust* (Lo et al., 2008).

We have developed an R package (*plateCore*) that also takes advantage of the functionality in *flowCore* and *flowViz* to create methods and data structures for processing large, plate-based FCM datasets. *plateCore* is not designed to be a GUI driven end-user tool, but rather to help develop a standardized platform for the analysis of FC-HCS data. These analyses often represent a collaborative effort between cytometry experts who generate the data and the quantitative individuals who help deal with the large volume information. In order for this collaboration to work, the cytometrists must have confidence in the results of the automated analysis. To this point, we demonstrate the equality of our results to those produced by an expert cytometrist using FlowJo.

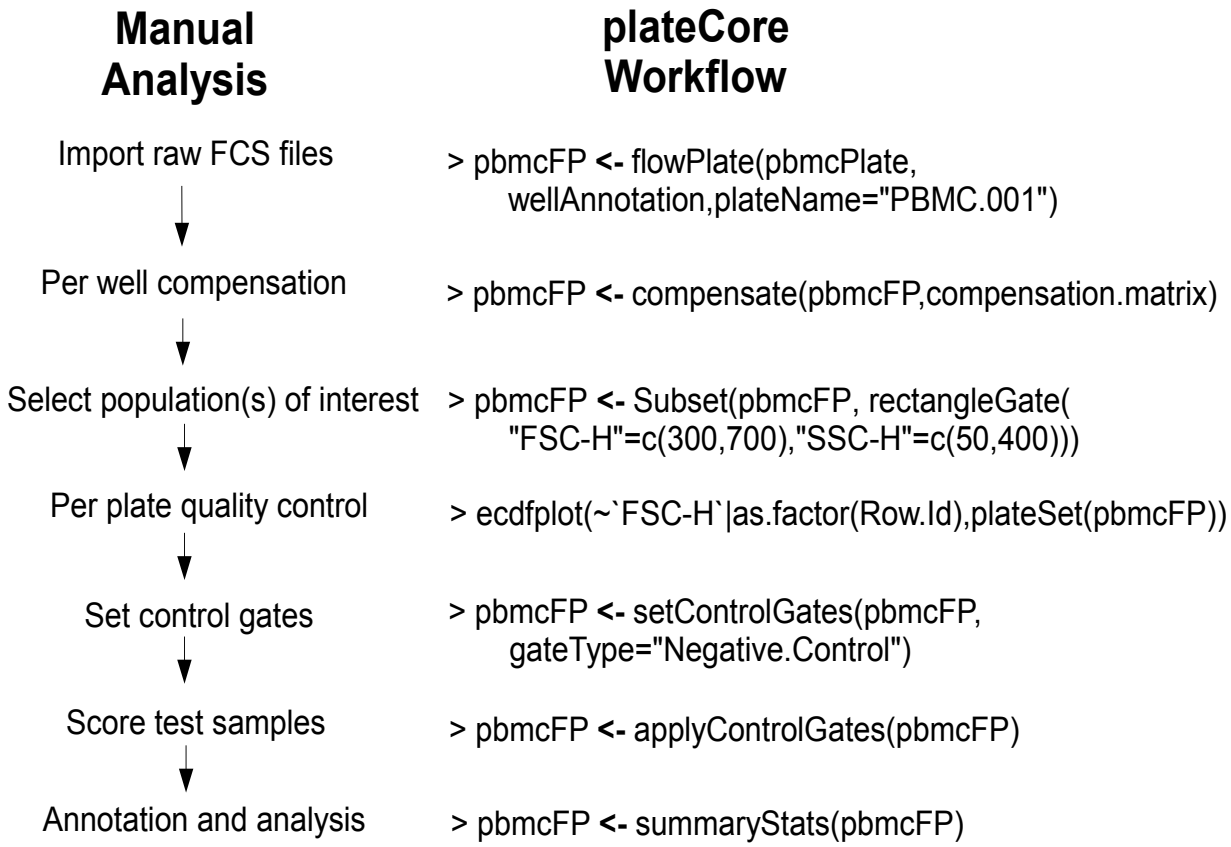

Figure 1: Typical FC-HCS plate workflow on the left and corresponding steps from a PBMC lymphocyte *plateCore* analysis on the right. Compared to analyses performed using existing GUI FCM tools, *plateCore* can reduce the level of subjectivity associated with creating the negative control gates and also makes it easier to aggregate multiple plates into an experiment level object for visualization and reporting. Providing *plateCore* scripts along with the raw FCM data for FC-HCS experiments helps to ensure that analysis is transparent and reproducible.

Materials and Methods

Data

The data analyzed in this study was part of the initial set of experiments used to validate the BD FACS CAP platform. BD FACS CAP was designed as a cell characterization tool to screen for the presence of a large number of different human cell surface markers, and it was important to show that the assay was able to correctly identify positive and negatively staining markers on a well studied cell population, such as PBMC lymphocytes. Previously frozen PBMC samples from two donors were analyzed on a BD FACS Calibur using BD FACS CAP staining plates. The analysis was performed on 96-well plates with 189 different antibodies arrayed three per well in 63 test wells, along with 30 isotype control wells and three unstained controls. The complete list of BD FACS CAP antibodies can be found at http://www.bd.com/technologies/discovery_platform/BD_FACS_CAP.asp. FCM files for the 5 plates (two for Donor 1 and three for Donor 2), are available for download from http://www.ficcs.org.

Analysis

The *plateCore* scripts used to perform the analysis are provided in supplementary materials. Briefly, the FCM files were first processed using a combination of static gates(`rectangleGate`) and data driven gates (using `norm2filter` and *flowCore*) to pick out the lymphocytes in the forward (FSC) and side scatter (SSC) channels. The quality of the data was then assessed by looking for fluidic events such as bubbles, pressure drops, or large aggregates that can shift the baseline fluorescence readings. Fluidic events can often be identified by plotting the empirical cumulative density (ecdf) plots of FSC values for each well, and looking for distributions shifted relative to other wells (Le Meur et al., 2007). Based on the ecdf plots, several wells were further investigated by cytometry experts who determined that the shifts were in an acceptable range. Next the threshold between positive and negative cells were determined using the isoytpe controls, which provided a gross estimate of non-specific binding in the primary antibodies. One-dimensional gates were created using using the isotype thresholds, and these gates were applied to identify cells that are positively stained for each marker.

An example of the progression from raw FCM data files to a completed *plateCore* analysis is shown in Figure 1. List mode FCS files for a single plate were read into a `flowSet` using *flowCore*, and then a `flowPlate` was created by integrating the plate annotation file with the `flowSet`. The `flowPlate` was then compensated, data quality was assessed, and gates were set according to a negative control. These control gates were then applied to test wells to find cells that had specific staining in channels of interest.

In addition to *plateCore*, the five PBMC plates were also analyzed using FlowJo. First, an analysis template was created where test wells and their corresponding isotype control well were assigned to one of 30 groups. Wells in each group had similar sets of

antibody-dye conjugates, and the expression threshold (*i.e.*, isotype gate) was initially set using the isotype control well. Data for each plate was imported into FlowJo using the template and lymphocytes were selected using a morphology (FSC-SSC) gate. Event data for the isotype well was then visualized on a log scale, and the expression threshold for each stained channel was set by picking a value that lies above the bulk of the events. For BD FACS CAP, the isotype gate are initially set so that approximately 1% or less of the events in the isotype well are above the threshold. These gates were then applied to the test wells, and the gates were moved up or down depending upon positive and negative test well populations. If the the population of cells in positive wells was much higher than the isotype gate, then the gate was moved up to help reduce false positives associated with non-specific staining. Similarly, if the isotype gate was higher than negative samples, the gate would be moved down to ensure that positive cells were classified correctly. The percentage of cells above the threshold for each of the 189 antibodies was then exported for each plate.

Results

Although this study focuses on comparing two different FC-HCS analysis methods, it is important to consider the orignal goal of the experiment used to generate the data when interpreting the results. BD FACS CAP was designed to provide a standard assay platform for screening a large number of markers on many different cell types. The validation effort for BD FACS CAP included running the assay on well-charaterized cell types to find markers with either positive or negative staining, and comparing these results to published cell expression profiles in the literature. The PBMC lymphocyte staining results presented in the following section represent one of the cell types used for validating the technology.

plateCore

Descriptions of marker expression profiles for particular cell populations in flow cytometry often use terms like positive-negative, or bright-dim, to qualify the amount of target present. We elected to take a more quantitative approach to reporting BD FACS CAP results by providing the percentage of cells that lie above the isotype gate and also the Median Fluorescence Intensity (MFI) ratio for each of the 189 markers. We define MFI ratio as the ratio of the median signal intensity for a specific antibody relative to the median signal of its corresponding isotype control. A cell population with 0% positive cells and an MFI ratio near 1 would traditionally be classified as negative, while a population with $\geq$99% positive cells and an MFI ratio $\geq$10 would be classified as positive or bright. Follow-up studies, including single color titrations and competition experiments of markers characterized using BD FACS CAP, show that high percent positive markers ($\geq$ 90%) are usually confirmed as positive, while staining in markers with a low percentage of positive cells ($\leq$ 10%) is often the result of non-specific binding (data not shown).

Note that these percentages refer to the fraction of cells above the isotype threshold, but this does not necessarily imply heterogeneous staining in multiple populations.

Estimates of the percentage of positive cells versus the MFI ratio made using *plateCore* for each of the 189 markers from the 5 plates are shown in Figure 2. There is a clear sigmodial relationship between the percentage of positive cells and the log of the MFI ratio, even though the different antibodies had been conjugated to different fluorophores (either Alexa 488, FITC, PE, PerCP, APC, or Alexa 647) and matched against different isotypes (either IgG1, IgG2, IgG2a, IgG2b, IgG3, or IgM). Points that are off the curve should be evaluated on a case-by-case basis to ensure that there are no problems with the test and control wells, and that the isotype gate settings are reasonable. Detailed results for each marker are not presented in this study, but since the majority of antibodies on the BD FACS CAP staining plate are known to bind different leukocytes, it is not surprising that a large fraction would be identified as positive on PBMCs. Markers such as CD44, CD45, CD47, and CD59 are broadly expressed on lymphocytes and were highly positive (>99%) in this study.

Additionally, visualizations such as Figure 2 suggest that it may be worthile to report a predicted percent positive value for each marker, which would be based on a regression of the observed percent positive to the log of the MFI ratio. Calculating the ratio of test well MFI versus the isotype control for a marker is obviously easier than developing an automated approach to isotype gating, and Figure 2 implies that the two approaches would produce similar estimates.

Comparison to FlowJo

Automating the creation and modification of isotype gates made by cytometrists analyzing BD FACS CAP data using FlowJo is challenging. Cytometrists adjust gates based on expert knowledge about the performance of specific antibody types and dyes, or after identifying positive or negative test samples. If the isotype gate cut off the bottom portion of a positive cell population, then the gate was moved down. Similarly, if the the isotype gate included too many cells from negative test wells, it was moved up.

The automated approach employed in *plateCore* determines the threshold using isotype controls. The gate (G_{ij}) for isotype i, channel j is set according to:

$$G_{ij} = \max(99\text{th}_{ij},\ \text{MFI}_{ij} + 4\text{MAD}_{ij}), \tag{1}$$

where 99th_{ij} is the 99th percentile for the fluorescence signal and MAD is Median Absolute Deviation on a linear scale. While this simple, non-parametric method works surprisingly well for BD FACS CAP, advances in model-based clustering methods, such as those in *flowClust*, should lead to future performance improvements in automated gating. Comparisons of the *plateCore* and FlowJo analysis are shown in Figure 3.

FlowJo and *plateCore* produced very similar percent positive estimates for markers that were clearly positive ($\geq$99) or negative (0%). Differences between the methods are more apparent when the isotype gate split the population of stained cells and the

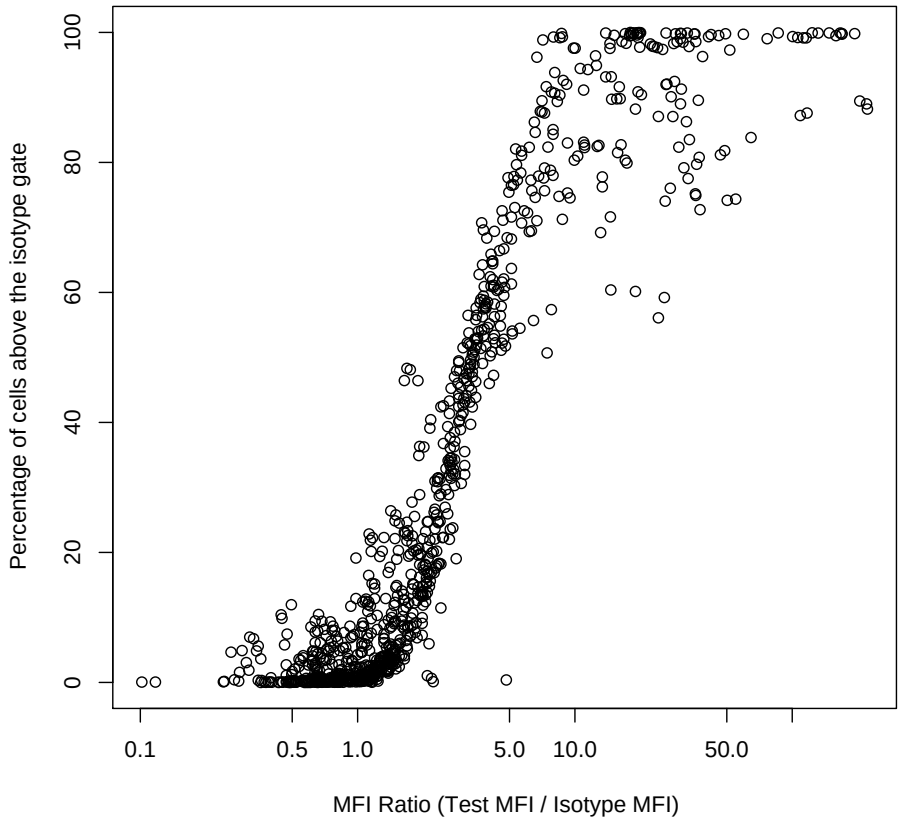

Figure 2: Plot of the Median Fluorescence Intensity (MFI) ratio for each of the 189 antibody-dye conjugates versus the percentage of positive events identified using plateCore from the five PBMC plates. The MFI ratio refers to the ratio of the test well MFI to the corresponding isotype control on a linear scale. Cell populations with MFIs that are close to the isotype control are often split by the isotype gate, reflecting the range of percent positive values for MFI ratios between 1 and 10.

percent positive estimates were between 30% and 70%. If the isotype gate is near the median signal for the marker, then small changes to the gate can dramatically change estimates of the percentage of positive cells. This same effect can be seen in Figure 2, where estimates of the percentage of positive cells rapidly increases as the MFI ratio goes from 1 to 10.

Examining in detail at one case where the *plateCore* and FlowJo methods disagree, such as CD98 on plate 9208 (Figure 4), highlights situations where our automated gating approach will fail. In this case the marker is 86% by FlowJo and 0% by *plateCore*. The isotype control for CD98 (well H08 in Figure 4) has >1% of its events in the FL1-H (FITC and Alexa 488) channel that are above the main population, resulting in the automated gate being set at the 99th percentile instead of the MFI+4MADS. Fortunately, this type of mistake can also be detected by looking the MFI ratio plot in Figure 2. CD98 for plate 9208 has an MFI ratio of 5 and a percent positive value of 0%, while other markers with a similar MFI ratio have values near 70%.

Donor Variation

In addition to identifying positively staining markers, another goal for this PBMC BD FACS CAP screen was to find markers that showed variation in their expression levels between the two donors. Simply performing a t-test on the percent positive estimates for the two donors resulted in a large number of markers with significant differences, even after correcting for multiple tests. Most of these differences are not biologically relevant, since they reflect the uncertainty in the placement of the isotype gate rather than differences in marker expression between the two donors. As shown in Figure 3, small changes to the isotype gate can have large effects on the estimates of the percentage of positive cells for values between 30% and 70%. These markers will be called differentially expressed, even though the signal levels for the two populations may be very close. Similarly, tests for differences in fluorescence signal intensities are also often significant due to the large number of events in cell population.

Instead of testing for differences in means, we instead used a graphical approach classify markers are candidates for donor variation and for further analysis. Variation in estimates of the percentage of positive cells was modeled using a binomial (n=5), where markers that are clearly positive or negative (0 or 100%) have little variation and those near the isotype gate (50%) are highly variable (Figure 5). Variation in percent positve estimates that exceeds what is expected from a binomial may represent biological differences in the donors. The expected and observed variations in the percentage of positively stained cells for the 189 markers in this study are shown in Figure 5. Discounting CD98, which had an unusual isotype control, the markers with the most variation (S.D. near 30%) were CD85, CD97, CD154, CD184, and CD252. Since CD85, CD97, CD154, and CD252 are associated with activated T-cells, it is tempting to speculate that this difference represents biological variation between the two donors as opposed to technical differences in the processing and gating of the cells.

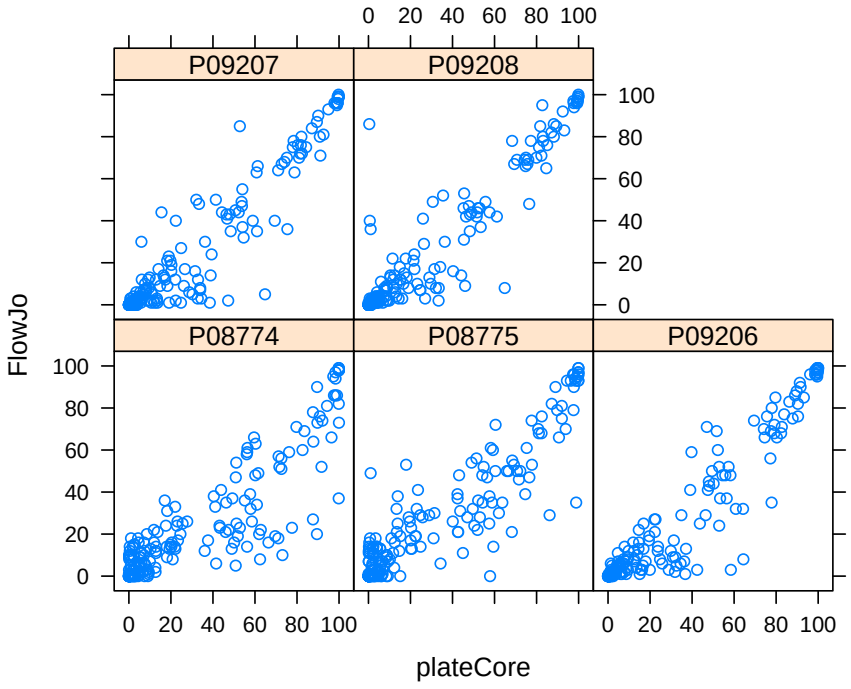

Figure 3: Percent positive results for 189 BD FACS CAP markers analyzed using either *plateCore* or FlowJo for the 5 PBMC plates. Markers that varied the most between the the two methods tended to have intermediate percent positive values (30% to 70%), reflecting the difficulty of gating populations whose MFI is near the isotype threshold. (%need to say what the P0907 labels mean, need mroe informative labels thatn just "'plate Cote and FlowJo (maybe jsut stick "'analysis) at the end. Again its confusing what positive vs 30% positive is. You said there was a threshold at 10% to get the label of positive. – Ryan)

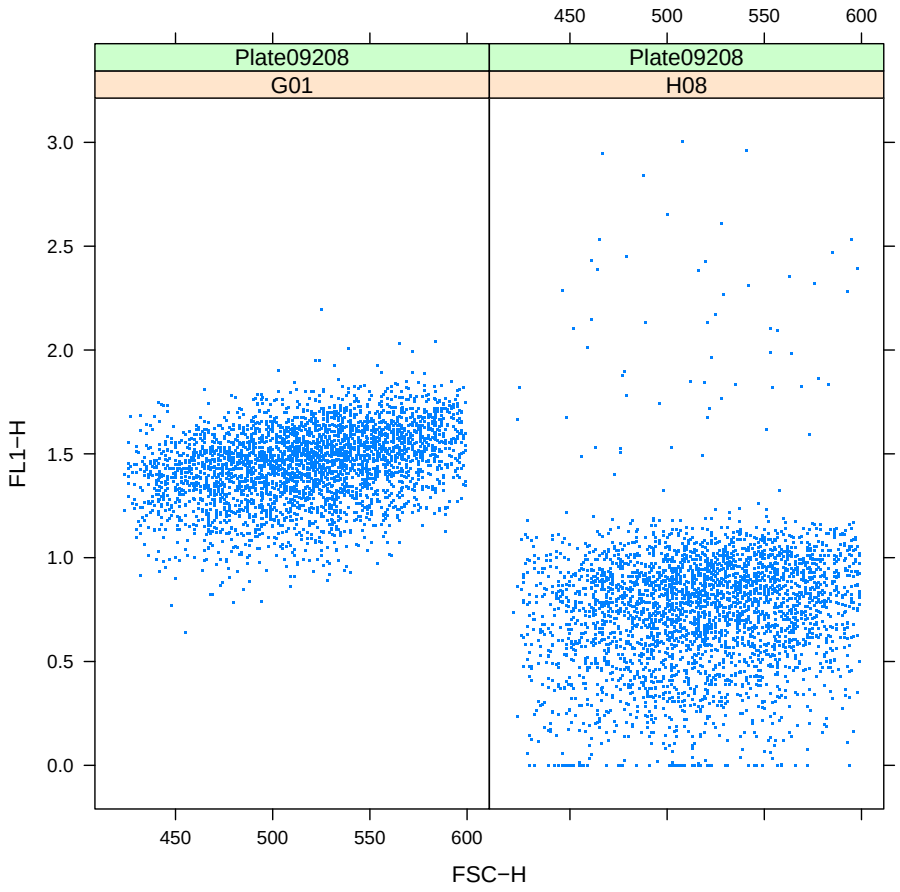

Figure 4: Dotplot for CD98 in well G01 and its isotype control well H08 for plate 9208. FlowJo (86%) and plateCore (0%) reported dramatically different percent positive values for this marker. The difference was caused by the isotype well having >1% of the events above the main population, which resulted in the automated gate being set at the 99th% instead of the MFI+4MADs. (indicate that G01 is a well ID PLate0909 is a plate ID - Ryan)

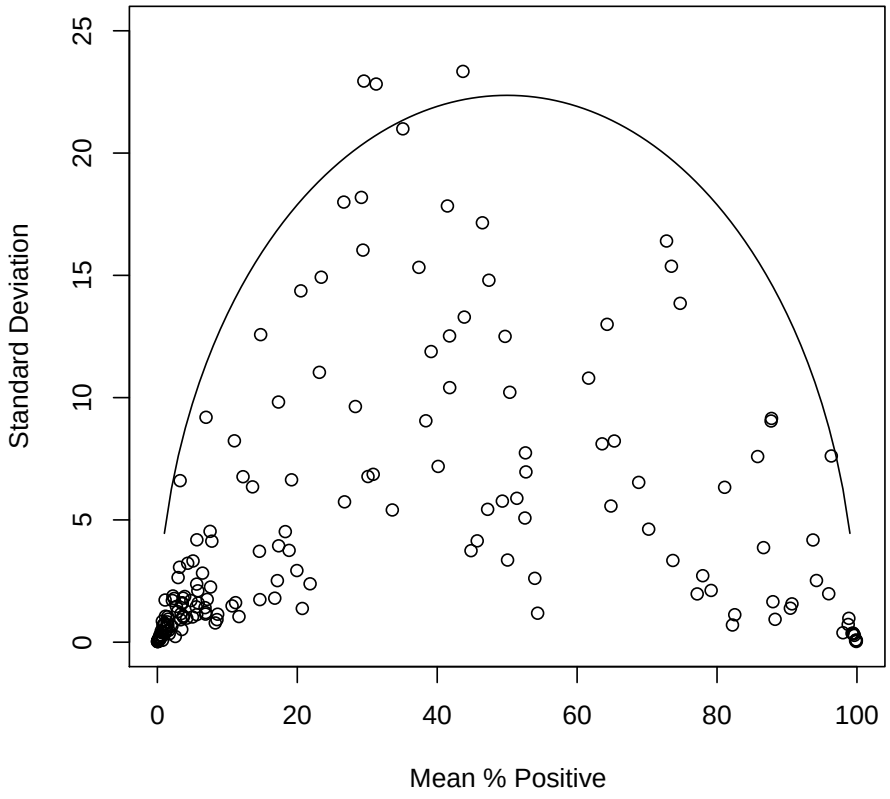

Figure 5: Scatterplot showing the mean percentage of positive cells for each positive marker versus the observed (circles) and expected (line) sample standard deviation. Expected values follow a binomial distribution with n=5. Markers that lie above the expected line will (%were? - Ryan) be further evaluated using titration and competition experiments to see if these results represent real variation between the two donors. The curve in the expected line reflects the difficulty in gating samples whose median signal (MFI) is near the isotype cutoff, since the percentage of positive cells calculated can shift dramatically with small changes in the gate. (standard deveiation of what? mean % positive of what? - Ryan))

Discussion

Our approach to this PBMC BD FACS CAP study relied on processing the raw data in parallel using both FlowJo and *plateCore*. FlowJo allowed the ctyometrists to thoroughly investigate individual wells, and gave them confidence that the *plateCore* results were correct (see Figure 3). Using *plateCore*, we were able to reduce the level subjectivity in setting isotype gates, eliminate mistakes associated with manual export and merging of plate output, and automate the creation of plots and data quality reports that summarized the experiment. Additionally, the *plateCore* scripts and experimental annotation can be shared with other cytometry groups, allowing them to reproduce our analysis.

In addition to subjective gating, the lack of a standard format for describing large FCM experiments also makes it difficult for anyone other than the original experimenter to replicate an analysis. (You could add something about MIFlowCyt here as well, as even if people had a format to follow, unless they put all the data in necessary to understand, just having a format isn't enough - Ryan) The development of mechanism to bundle experimental metadata descriptions with FCS data files should make it easier to access metadata in future FCM studies, but currently this information is typically provided either as spreadsheet or a pictorial layout of a 96 well plate. Since the creation of `flowPlate` requires users to make a standard sample annotation file, plate layouts from *plateCore* can then be easily shared along with the raw FCS2.0/3.0 files. The standard format for *plateCore* sample annotations provides a convenient way to manage the plate metadata associated with complex FC-HCS experiments.

While this same analysis can be performed relatively quickly in other FCM software packages, it can be difficult to reproduce the gating decisions made by a single expert user. (Expand - Ryan)

Markers that are expressed on a small subset of lymphocytes, or markers that are dimly expressed, would not be found with this screening approach. (Expand - Ryan)

Since, BD FACS CAP was designed as screening tool to identify markers for further analysis, false negatives were a bigger concern than false positives. The plateCore settings were chosen to err on the side of calling samples positive. (Why, make it clear to uninformed readers by stating explicitly? what about for other studies? more discussion here - Ryan)

The complexity of large FCM experiments, like BD FACS CAP, highlight the difficulty of applying existing FCM analysis platforms to high-throughput studies. Generating and interpreting results from this PBMC study required extensive collaboration between flow cytometrists, bioinformaticians, and statisticians. At various points in the analysis, each group needed to access the raw data, annotation, and details about the experimental design. Providing this access using stand-alone FCM platforms is expensive in terms of the price of multiple software licenses and in time spent training statisticians and bioinformaticians to use the programs. Fortunately the Bioconductor FCM packages are modeled on standard data structures used for microarrays, which should al-

ready be familiar to most quantitative individuals working on high-throughput biological problems. We found that *flowCore*, *flowViz*, and *plateCore* provided an open analysis platform that facilitated communication between the flow cytometrists generating the data, and the computational experts analyzing the data.

References

R. Gentleman, F. Hahne, J. Kettman, and N. Le Meur. Bioconductor package flowq. URL `http://www.bioconductor.org`.

Robert C Gentleman, Vincent J. Carey, Douglas M. Bates, Ben Bolstad, Marcel Dettling, Sandrine Dudoit, Byron Ellis, Laurent Gautier, Yongchao Ge, Jeff Gentry, Kurt Hornik, Torsten Hothorn, Wolfgang Huber, Stefano Iacus, Rafael Irizarry, Friedrich Leisch, Cheng Li, Martin Maechler, Anthony J. Rossini, Gunther Sawitzki, Colin Smith, Gordon Smyth, Luke Tierney, Jean Y. H. Yang, and Jianhua Zhang. Bioconductor: Open software development for computational biology and bioinformatics. *Genome Biology*, 5:R80, 2004. URL `http://genomebiology.com/2004/5/10/R80`.

F. Hahne, N. LeMeur, R.R. Brinkman, B. Ellis, P. Haaland, D. Sarkar, J. Spidlen, E. Strain, and R. Gentleman. flowcore a bioconductor package for high throughput flow cytometry. *BMC Bioinformatics*, 2009.

N. Le Meur, A. Rossini, M Gasparetto, C Smith, R.R. Brinkman, and R. Gentleman. Quality assessment of ungated flow cytometry data in high throughput experiments. *Cytometry A*, 71:393–403, 2007.

K. Lo, R. R. Brinkman, and R. Gottardo. Automated gating of flow cytometry data via robust model-based clustering. *Cytometry A*, 73A:321–332, 2008.

Holden T Maecker, Aline Rinfret, Patricia D'Souza, Janice Darden, Eva Roig, Claire Landry, Peter Hayes, Josephine Birungi, Omu Anzala, Miguel Garcia, Alexandre Harari, Ian Frank, Ruth Baydo, Megan Baker, Jennifer Holbrook, Janet Ottinger, Laurie Lamoreaux, C. Lorrie Epling, Elizabeth Sinclair, Maria A Suni, Kara Punt, Sandra Calarota, Sophia El-Bahi, Gailet Alter, Hazel Maila, Ellen Kuta, Josephine Cox, Clive Gray, Marcus Altfeld, Nolwenn Nougarede, Jean Boyer, Lynda Tussey, Timothy Tobery, Barry Bredt, Mario Roederer, Richard Koup, Vernon C Maino, Kent Weinhold, Giuseppe Pantaleo, Jill Gilmour, Helen Horton, and Rafick P Sekaly. Standardization of cytokine flow cytometry assays. *BMC Immunol*, 6:13, 2005. doi: 10.1186/1471-2172-6-13. URL `http://dx.doi.org/10.1186/1471-2172-6-13`.

D. Sarkar, N. Le Meur, and R. Gentleman. Using flowViz to visualize flow cytometry data. *Bioinformatics*, 24(6):878, 2008.